ISSN 0206-5657. Вісник Львівського університету. Серія біологічна. 2016. Випуск 73. С. 41–46
Visnyk of the Lviv University. Series Biology. 2016. Issue 73. P. 41–46

УДК 576.34: 576.311.34

THE DEGRADATIVE INACTIVATION OF THE CYTOSOLIC ENZYME FRUCTOSE-1,6-BISPHOSPHATASE UPON THE GLUCOSE ADAPTATION OF THE METHANOL-GROWN YEAST *PICHIA PASTORIS*

N. Bulbotka[1*], K. Levkiv[1], O. Dmytruk[1], A. Sibirny[1,2]

[1] Department of Molecular Genetics and Biotechnology
Institute of Cell Biology, NAS of Ukraine
14/16, Drahomanov, Lviv 79005, Ukraine
[2]Department of Biotechnology and Microbiology, Rzeszow University
4, Zelwerowicz St., Rzeszow 35-601, Poland
e-mail: ninabulbotka@ukr.net

Methylotrophic yeasts can selectively use substrates from a mixture of different carbon sources, but glucose being the preferred ones. Shift of methanol-grown cells into a glucose containing medium leads to fast inactivation and degradation of peroxisomal and cytosolic enzymes of methanol metabolism. This phenomenon is known as catabolite inactivation which can occur due to proteolytic degradation. Degradation of peroxisomal enzymes occurs due to the autophagic degradation (pexophagy) in methylotrophic yeast whereas mechanisms of degradation and inactivation of cytosolic enzymes of methanol metabolism remain unknown. We studied inactivation and degradation of cytosolic enzyme fructose-1,6-bisphosphatase (FBPase) versus peroxisomal alcohol oxidase (AOX). Degradation of FBPase was defective in the mutant of *Pichia pastoris* SMD1163 (*pep4 prb1*) defective in vacuolar proteinases suggesting the role of vacuoles in degradation of cytosolic enzyme of methanol metabolism. Inactivation and degradation of FBPase was also strongly retarded in Δgss1mutant defective in glucose sensor suggesting the role of glucose signaling in these processes.

Keywords: methylotrophic yeast, fructose-1,6-bisphosphatase, catabolite inactivation, proteolytic degradation.

The methylotrophic yeast *Pichia pastoris* has become an important host organism for recombinant protein production [10]. The success of methylotrophic yeasts in the production of recombinant proteins is highly linked to the very strong and tightly regulated promoters of some genes of the methanol utilization pathway (MUT pathway) [9]. Proteolytic degradation has been a perpetual problem when yeasts are employed to express recombinant proteins. However, no in-depth analysis on the conditions that promote proteolysis or the nature of the proteases acting on the desired protein are exactly known [16]. To obtain strains defective in proteolysis of heterologous proteins we should explore proteolysis mechanisms of cytosolic proteins in methylotrophic yeasts. Two alternative mechanisms for the proteolysis have been described: (a) degradation by vacuolar proteases – autophagy, and (b) ubiquitin-dependent degradation – proteasomal degradation [4].

Inactivation and degradation of cytosolic enzymes on example of fructose-1,6-bisphosphatase (FBPase) have been most extensively studied in backer's yeast [15]. When *Saccharomyces cerevisiae* cells are cultivated in media containing a nonfermentable carbon source, glucose is synthesized via the gluconeogenic pathway. Shifting these cells to glucose-containing media leads to a rapid switch from gluconeogenesis to glycolysis. During this metabolic adaptation, the key regulatory gluconeogenetic enzyme, FBPase [6], is rapidly inactivated and then degraded in a process called catabolite inactivation. This inactivation process consists of two separate steps:

1) phosphorylation of the enzyme and 2) degradation of the protein [15]. The catabolite repression by glucose and related sugars is an example of the quite different regulatory mechanisms, which includes transcription repression and control of translation by the RNA-binding proteins [7]. It was shown that the glucose-induced degradation of FBPase in backer's yeast occurred both in the proteasome and in the vacuole, depending on the growth conditions. For example, when cells were starved for a short period of time (for 1 day) and then shifted to glucose, FBPase was degraded in the proteasome. However, when glucose was added to cells that have been starved for longer periods of time (for 3 days), FBPase was degraded in the vacuole [1, 2]. Since pathways of cytosolic enzymes inactivation and degradation in methylotrophic yeast remain unknown, we studied these processes on example of FBPase in *P. pastoris*.

Materials and methods

The wild type strain of *Pichia pastoris* GS200 *his4 arg4* [3], the strain with both defective vacualar proteinases *pep4* and *prb1* SMD1163 [18] and also the Δ*gss1*strain with deletion of a gene coding a glucose sensor, Gss1p were used in this research. Last one was obtained by the replacement of the ORF of *GSS1* by the *ScARG4* [14]. Rich YPD medium contained 0.5% yeast extract, 1.5% peptone, and 2% glucose. Synthetic minimal medium contained carbon source (1% methanol or 2% glucose), 0.17% yeast nitrogen base without amino acids and ammonium sulfate (YNB), 0.5% ammonium sulfate, and 40mg/l of amino acids (histidine, arginin) if needed. All strains were grown at 30 °C, 220 rpm. Solid media contained 2% agar.

Cells were grown in methanol containing media for 1 day or 3 days to induce FBPase and peroxisomal alcohol oxidase (AOX). Then cells were shifted to fresh glucose containing medium without Nitrogen source and with special proteasome inhibitor MG132 (carbobenzoxyl-L-leucyl-L-leucyl-L-leucine) or without it. Cells were collected on 0, 6 and 24 hours of glucose adaptation by centrifugation (3500 rpm, 10 min), disintegrated in 50mM potassium phosphate buffer pH 7.0 with equal volume of glass beads (15 min, 4°C) and after dialysis cell free extract was used for the further investigation. The obtained protein samples were used for Western blot analysis after protein content determination by the Lowry method [13]. SDS-PAGE and immunoblotting were performed as described previously [11, 12]. We used antibodies against the human FBPase and against the AOX of *P. pastoris.* Antigen-antibody complexes were detected by enhanced chemiluminescence. The FBPase activity was assayed as in Gancedo J. et al. 1971. [5] with some modifications. Alcohol oxidase activity was determined by the ABTS/POD (2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)/peroxidase) method as described in [17].

Results and discussion

As it was mentioned before, the *Saccharomyces cerevisiae* FBPase is degraded by the proteasome-dependent pathway after glucose starvation of the yeasts for 1 day and by the vacuole-dependent pathway (autophagy) after glucose starvation of the cells for 3 days. In this work, we studied inactivation and degradation of cytosolic enzyme FBPase versus peroxisomal AOX in the methylotrophic yeast *P. pastoris*. To inhibit proteasome-dependent degradation of FBPase, we applied the peptide-aldehyde proteasome inhibitor MG132 which inhibits 20S proteasome activity by covalently binding to the active site of the beta subunits and effectively blocks the proteolytic activity of the 26S proteasome complex [8].

It was found that inactivation of the FBPase insignificantly was inhibited by proteasomal inhibitor MG-132 in all strains. According to the levels of FBPase upon short- and long- time starvation conditions we suggest that inactivation of FBPase didn't depend on the duration of glucose starvation in methylotrophic yeasts *P. pastoris* in contrast to baker's yeasts (Fig. 1, 2). Studying the inactivation of peroxisomal enzyme AOX was used as a control.

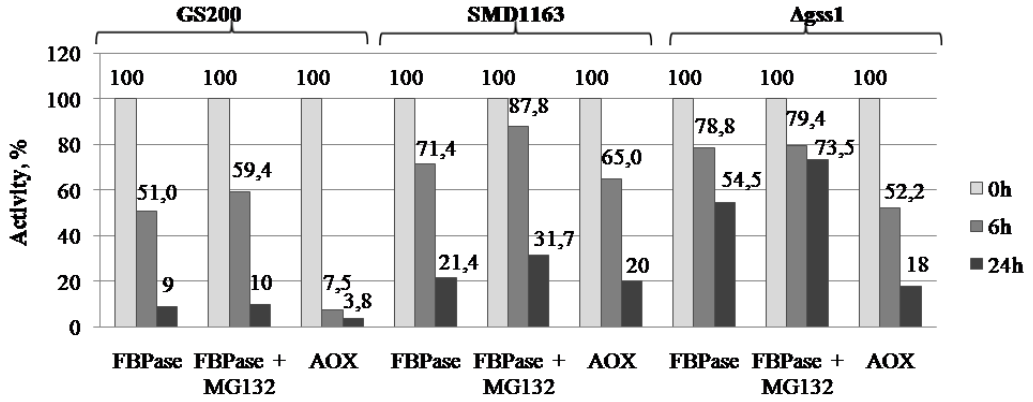

Fig. 1. Inactivation of cytosolic enzyme FBPase versus peroxisomal AOX in wild type strain GS200, SMD1163 and Δ*gss1* after short term starvation (1 day)

The FBPase and AOX inactivation on 24 hour of glucose adaptation after 1 or 3 days incubation in methanol containing medium in proteinases defective SMD1163 strain was retarded in contrast to that in the wild-type strain. Therefore we suggest that normal functioning of vacuoles has higher impact into inactivation of FBPase than that of proteasome, especially as addition of MG132 had insignificant influence on FBPase inactivation. Retardation in FBPase and AOX inactivation in Δ*gss1* strain can be caused by damaged glucose recognition and/or changes in glucose transport and therefore further changes in processes involved in glucose catabolite inactivation (Fig. 1, 2).

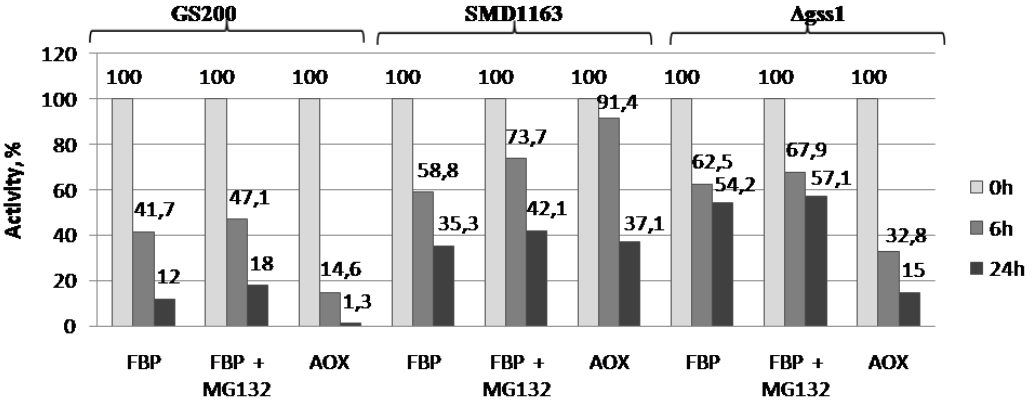

Fig. 2. Inactivation of FBPase versus AOX in GS200, SMD1163 and Δ*gss1* after long term starvation (3 days)

The results of Western blot analysis were correlated with enzyme inactivation data. Western blotting analysis showed that during inactivation of FBPase, degradation of the corresponding protein occurs and proteasomal inhibitor MG-132 only slightly inhibited this process (Fig. 3).

It is known that AOX is degraded by autophagy and its degradation in strain SMD1163 is suppressed due to defects of vacuolar proteinases. At the same time, degradation of FBPase was also damaged in this strain suggesting the role of vacuoles in degradation of this enzyme. Degradation of FBPase was also strongly retarded in Δgss1 mutant defective in glucose sensor suggesting the role of glucose signaling in this process (Fig. 3).

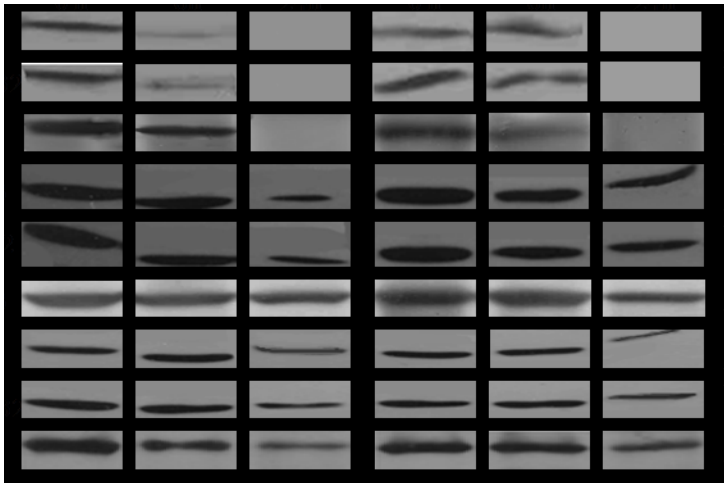

Fig. 3. The Western blotting of FBPase degradation using human Fbp1 antibodies in the wild-type strain GS200, SMD1163 and *Δgss1* with and without addition of proteasomal inhibitor MG-132

Thus, the glucose signaling is involved both in the inactivation and degradation of cytosolic enzymes and in pexophagy. Degradation of cytosolic enzyme FBPase are occurred by mainly in vacuolar pathways independently on the duration of glucose starvation of the methylotrophic yeast in contrast to the baker's yeast.

REFERENCES

1. *Alibhoy A., Giardina B., Dunton D., Chiang H.* Vps34p is required for the decline of extracellular FBPase in the vacuole import and degradation pathway // J. Biol. Chem. 2012. Vol. 287. №. 39. P. 33080–33093.
2. *Brown C. R., Wolfe A., Cui D., Chiang H.* The vacuolar import and degradation pathway merges with the endocytic pathway to deliver FBPase to the vacuole for degradation // J. Biol. Chem. 2008. Vol. 283. №. 38. P. 26116–26127.
3. *Cregg J. M. Barringer K. J., Hessler A. Y., Madden K. R. Pichia pastoris* as a host system for transformations // Mol. Cell Biol. 1985. Vol. 5. №. 12. P. 3376–3385.
4. *Gancedo J.* Yeast carbon catabolite repression // Microbiol. Mol. Biol. Rev. 1998. Vol. 62. № 2. P. 334–361.
5. *Gancedo J. M., Gancedo, C.* Fructose-1,6-diphosphatase, phosphofructokinase and glucose-6-phosphat dehydrogenase from fermenting non fermenting yeasts // Arch. Microbial. 1971. Vol. 76. P. 132–138.
6. *Giardina B. J., Chiang H. L.* The key gluconeogenic enzyme FBPaseis secreted during prolonged glucose starvation and is internalized following glucose re-feeding via the non-classical secretory and internalizing pathways in *Saccharomyces cerevisiae* // Plant Signal. Behavior. 2013. Vol. 8. №8.
7. *Görke B., Stülke J.* Carbon catabolite repression in bacteria: many ways to make the most out of nutrients // Nat. Rev. Microbiol. 2008. Vol. 6. № 8. P. 613–624.
8. *Guo N., Peng Zh.* MG132, a proteasome inhibitor, induces apoptosis in tumor cells // Asia Pac. J. Oncol. 2013. Vol. 9. P.6–11.
9. *Hartner F., Glieder A.* Regulation of methanol utilisation pathway genes in yeasts // Microb. Cell Factor. 2006. Vol. 5. P. 39–59.

10. *Krainer F., Dietzsch K., Hajek T. et al.* Recombinant protein expression in *Pichia pastoris* strains with an engineered methanol utilization pathway // Microb. Cell Factor. 2012. Vol. 11. P. 22–35.
11. *Kyhse-Andersen J.* Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose // J. Biochem. Biophys. Methods. 1984. Vol. 10. P. 203–209.
12. *Laemmli U. K.* Cleavage of structural proteins during the assembly of the head of bacteriophage T4 // Nature. 1970. Vol. 227. P. 680–685.
13. *Lowry O., Rosebrough N., Farr L., Randal R.* Protein measurement with the Folin phenol reagent //J. Biol. Chem. 1952. Vol. 193. P. 265–275.
14. *Polupanov A., Nazarko V., Sibirny A.* Gss1 protein of the methylotrophic yeast *Pichia pastoris* is involved in glucose sensing, pexophagy and catabolite repression // Int. J. Biochem. Cell Biol. 2012. Vol. 44. №. 11. P. 1906–1918.
15. *Regelmann J., Schule T., Josupeit F. et al.* Catabolite degradation of FBPasein the yeast *Saccharomyces cerevisiae*: a genome-wide screen identifies eight novel *GID* genes and indicates the existence of two degradation pathways // Mol. Biol. Cell. 2003. Vol. 14. P. 1652–1663.
16. *Sinha J., Plantz B., Inan M. et al.* Causes of proteolytic degradation of secreted recombinant proteins produced in methylotrophic yeast *Pichia pastoris*: case study with recombinant ovine interferon-T // Biotech. Bioengineer. 2005. Vol. 89, № 1. P. 102–112.
17. *Tani, Y., Sakai, Y., Yamada H.* Isolation and characterization of a mutant of a methanol yeast, *Candida Boidinii* S2, with higher formaldehyde productivity //Agric. Biol. Chem.1985. Vol. 49. P. 2699–1706.
18. *Tuttle D. L., Dunn Jr. W. A.* Divergent modes of autophagy in the methylotrophic yeast *Pichia pastoris* // J. Cell Sci. 1995. Vol. 108. P. 25–35.

Стаття: надійшла до редакції 26.07.16
доопрацьована 31.08.16
прийнята до друку 2.09.16

ДЕГРАДАЦІЙНА ІНАКТИВАЦІЯ ЦИТОЗОЛЬНОГО ФЕРМЕНТУ ФРУКТОЗО-1,6-БІФОСФАТАЗИ ЗА УМОВ ГЛЮКОЗНОЇ АДАПТАЦІЇ У МЕТАНОЛВИРОЩЕНИХ ДРІЖДЖІВ *PICHIA PASTORIS*

Н. Булботка[1], К. Левків[1], О. Дмитрук[1], А. Сибірний[1, 2]

[1] Відділ молекулярної генетики та біотехнології
Інституту біології клітини Національної академії наук України
вул. Драгоманова, 14/16, Львів 79005, Україна
[2] Відділ біотехнології і мікробіології, Жешувський університет
вул. Зельверовича, 4, Жешув 35-601, Польща
e-mail: ninabulbotka@ukr.net

Метилотрофні дріжджі можуть вибірково використовувати субстрати зі суміші різних джерел Карбону, але глюкоза і фруктоза є більш вигідними. Перенесення клітин, вирощених на метанолі, в середовище, яке містить глюкозу, призводить до швидкої інактивації та деградації пероксисомних і цитозольних ферментів метаболізму метанолу. Це явище відоме як катаболітна інактивація, що може відбуватися через протеолітичну деградацію. Деградація пероксисомних ферментів відбувається

ISSN 0206-5657. Вісник Львівського університету. Серія біологічна. 2016. Випуск 73

внаслідок деградації шляхом автофагії (пексофагії) у метилотрофних дріжджів, тоді як механізми деградації та інактивації цитозольних ферментів залишаються невідомими. Ми вивчали інактивацію і деградацію цитозольного ферменту фруктозо-1,6-біфосфатази (ФБФ-ази) порівнянно з пероксисомною алкогольоксидазою (АО). Деградація ФБФ-ази була пошкоджена в мутанта *Pichia pastoris* SMD1163 (*pep4 prb1*) з дефектом вакуолярних протеїназ, що свідчить про роль вакуолей у деградації цитозольних ферментів метаболізму метанолу. Інактивація і деградація ФБФ-ази також значно сповільнена у мутанта Δgss1 з дефектом сенсора глюкози, що може свідчити про роль сигналізації глюкози в даному процесі.

Ключові слова: метилотрофні дріжджі, фруктозо-1,6-біфосфатаза, інактивація, деградація.

ДЕГРАДАТИВНАЯ ИНАКТИВАЦИЯ ЦИТОЗОЛЬНОГО ФЕРМЕНТА ФРУКТОЗО-1,6-БИФОСФАТАЗЫ В УСЛОВИЯХ ГЛЮКОЗНОЙ АДАПТАЦИИ У МЕТАНОЛВЫРАЩЕННЫХ ДРОЖЖЕЙ *PICHIA PASTORIS*

Н. Булботка[1], Е. Левкив[1], Е. Дмитрук[1], А. Сибирный[1,2]

[1] Отдел молекулярной генетики и биотехнологии
Института биологии клетки Национальной академии наук Украины
ул. Драгоманова, 14/16, Львов 79005, Украина
[2] Отдел биотехнологии и микробиологии, Жешувский университет
ул. Зельверовича, 4, Жешув 35-601, Польша
e-mail: ninabulbotka@ukr.net

Метилотрофные дрожжи способны выборочно использовать субстраты из смеси различных источников углерода, но глюкоза и фруктоза являются предпочтительными. Перенос клеток, выращенных в среде с метанолом, в среду, содержащую глюкозу, приводит к быстрой инактивации и деградации пероксисомних и цитозольных ферментов метаболизма метанола. Это явление известно как катаболитная инактивация, которая может происходить посредством протеолитической деградации. Деградация пероксисомних ферментов происходит вследствие аутофагии (пексофагии) в метилотрофных дрожжах, тогда как механизмы деградации и инактивации ферментов цитоплазмы остаются неизвестными. Мы изучали инактивацию и деградацию цитозольного фермента фруктозо-1,6-бифосфатазы (ФБФ-азы) по сравнению с пероксисомальной алкогольоксидазой (АО). Деградация ФБФ-азы была повреждена у мутанта *Pichia pastoris* SMD1163 (*pep4 prb1*) с дефектами вакуолярных протеиназ, что свидетельствует о роли вакуолей в деградации цитозольных ферментов метаболизма метанола. Инактивация и деградация ФБФ-азы также сильно замедлена у мутанта Δgss*1* с дефектным рецептором глюкозы, предполагая роль глюкозной сигнальной трансдукции в этом процессе.

Ключевые слова: метилотрофные дрожжи, фруктозо-1,6-бифосфатаза, инактивация, деградация.